REMARKS

Applicants respectfully requests entry of the amendments and remarks submitted herein. Claims 1, 4, 7 and 8 have been amended, claims 1-3, 5-6 and 51-53 have been canceled, and claims 9-50 have been withdrawn. Therefore, claims 1, 4, 7, 8 and 9-50 are currently pending.

The 35 U.S.C. §112 Rejection

Claims 1 and 8, as amended, are rejected under 35 U.S.C. §112, first paragraph, as failing to comply with the written description requirement. The examiner has indicated that the specification does not provide an adequate written description as to the numerous amino acid residues that can be substituted in at least one position of the residues 7, 23, and 9 of the Fn3 molecule.

Applicant asserts that the specification as originally filed provides adequate written description support for the claimed invention. Amended claim 1 recites a modified fibronectin type III tenth fibronectin unit (Fn3fn10) molecule comprising a stabilizing mutation of at least one residue involved in an unfavorable electrostatic interaction as compared to a wild-type Fn3, wherein the stabilizing mutation is a substitution of at least one of Asp 7, Asp 23 or Glu 9 with another amino acid residue.

Applicant may show adequate written description by demonstrating that an invention is complete by disclosure of sufficiently detailed, relevant identifying characteristics which provide evidence that applicant was in possession of the claimed invention, *i.e.*, complete or partial structure, other physical and/or chemical properties, functional characteristics when coupled with a known or disclosed correlation between function and structure, or some combination of such characteristics. *Enzo Biochem. v. Gen-Probe Inc.*, 323 F.3d 956, 963, 63 U.S.P.Q.2d 1609, 1613 (Fed. Cir. 2002). What is conventional or well known to one of ordinary skill in the art need not be disclosed in detail. *Hybritech Inc. v. Monoclonal Antibodies, Inc.*, 802 F.3d 1367,1384, 231 U.S.P.Q. 81, 94 (Fed. Cir. 1986). If a skilled artisan would have understood the inventor to be in possession of the claimed invention at the time of filing, even if every nuance of the claims is not explicitly described in the specification, then the adequate description requirement is met.

To the extent that the Examiner's statement is intended as a rejection that the amended claims encompass inoperative embodiments, the Examiner is requested to note that claims are in accord with the requirements of 35 U.S.C. § 112 if one of skill in the art, guided by the specification, could avoid inoperable combinations and practice the invention without undue experimentation. The mere possibility that a claim embraces inoperable embodiments does not render it unduly broad. In addition, it is not a function of the claims to specifically exclude all possible inoperative substances.

The chemical structure of the wild-type fibronectin type III tenth fibronectin unit (Fn3fn10) is well-known (see, *e.g.*, Main *et al.* 1992, of record). The claimed invention has a modification of this basic structure, which is a substitution of at least one of Asp 7, Asp 23 or Glu 9. Applicant provides functional characteristics of the claimed invention, namely, that the mutation is a stabilizing mutation of at least one of these three listed amino acid residues. A stabilizing mutation is defined in the specification at page 6, lines 20-24, as "a modification or change in the amino acid sequence of the Fn3 molecule, such as a substitution of one amino acid for another, that increases the melting point of the molecule by more than 0.1°C as compared to a molecule that is identical except for the change."

Applicant asserts that once he determined that residues Asp 7, Asp 23 and Glu 9 of the Fn3 molecule were the amino acids contributing to unfavorable intra-molecular electrostatic interaction as compared to the wild-type Fn3, one of ordinary skill in the art would know what amino acid residues could be substituted at these positions to enhance the stability of the Fn3. One of skill in the art would know that both Asp and Glu have a negative charge, and that the introduction of an amino acid that has either a neutral or positive charge would remove the unfavorable electrostatic interaction at residues 7, 23 and 9. Given the knowledge and level of skill in the art, a skilled person would immediately envisage the appropriate amino acids that could substitute for Asp 7, Asp 23 and Glu 9 of the Fn3 molecule, so that the Fn3 molecule is made more stable.

The structures and electrostatic properties of amino acids are fundamental knowledge in the field of protein biochemistry. The interactions of electrically charged particles within molecules are determined by the laws of classical electrostatics. Coulomb's law, which determines electrostatic energy (work) for charged particles, clearly indicates that placing two

negatively charged particles, such as the side chains of Asp and Glu, in close proximity is energetically unfavorable (See, *e.g.*, Voet & Voet, Biochemistry, third edition, Wiley (2004), pages 258-259, copy enclosed for the examiner's convenience). Coulomb's law also indicates that such electrostatic repulsions can be eliminated by (i) removing a charge by a mutation or (ii) by replacing a charged particle with a particle having the opposite charge. Therefore, once Applicant has identified the residues that cause unfavorable electrostatic interactions (*i.e.*, repulsions), one skilled in the art can design a mutation that reduces or eliminates the repulsions to generate a more stabile FN3fn10 molecule. Based on the fundamental knowledge in protein biochemistry, such a person can rationally design a mutation by replacing a residue in the triad with either a neutral amino acid or a positively charged amino acid. Applicant provides adequate discussion of tests to determine the stability of a modified molecule. See, Example XIX of the specification (pages 66-68). Therefore, Applicant has provided adequate written description as he has disclosed in sufficiently detail the relevant identifying characteristics that provide evidence that applicant was in possession of the claimed invention at the time the application was filed.

Page 3 of the Office Action cites to pages 76-77 of the specification to support the proposition that an electrostatic interaction can present an unpredictable effect in any Fn3 molecule. To address this concern, it should be noted that the claims have been amended to recite "Fn3fn10." Therefore, the discussion of other Fn3 molecules is no longer applicable. Regarding the passage at page 76, lines 19-20 of the specification ("Therefore, it is not clear why these destabilizing residues are almost completely conserved in FNfn10."), it is evident from the context of the sentence that the passage is referring to the general lack of understanding as to why <u>in nature</u> the Asp 7, Asp 23 and Glu 9 triad has not evolved into a more stable state. The examiner appears to be interpreting this passage as meaning that the electrostatic effect of the interactions of the residues is unpredictable. To the contrary, the electrostatic interactions of specific residues can be readily determined by one of skill in the art, and provide a logical place for one of skill to modify the proteins to make them more stable, <u>once a researcher determines which residues to investigate in a molecule</u>.

Regarding the Dao-pin reference, Applicants cited this reference for the proposition that substitutions of positively charged residues for other residues do not necessarily have a

stabilizing effect on a protein. Applicant was responding to the assertion by the examiner that "one having ordinary skill in the art would have known that positively charged residues such as Lys or Arg are known to have stabilizing effect on molecules such as Fn3 as taught by Koide [WO 98/56915]" (Office Action dated October 2, 2003, page 7). Applicant respectfully submits that this assertion by the examiner is not supported. One of skill in the art would know that introducing a Lys or Arg randomly to a protein does not always stabilize it. One of skill in the art would know that the stability of the engineered molecule depends upon what is the charge of a particular residue in the native molecule. For example, if the original residue has a positive charge, then simply substituting the original residue with another positively-charged residue (*i.e.*, a conserved substitution) will not necessarily render the molecule more stable. Conversely, if the original positively-charged residue is substituted with a neutral or negatively-charged residue, it may increase the stability of the molecule. Applicant asserts that once he determined that residues Asp 7, Asp 23 and Glu 9 of the Fn3 molecule were the amino acids contributing to unfavorable intra-molecular electrostatic interaction in the wild-type Fn3fn10, one of ordinary skill in the art would know what amino acid residues could be substituted at these positions to enhance the stability of the Fn3fn10.

It was found in the Dao-pin reference that the introduction of an attractive electrostatic interaction had a small effect on protein stability. The teachings of Dao-pin, however, are not in conflict with the present claimed invention. Instead, Dao-pin supports the inventiveness of the claimed invention. One of skill in the art, having read Dao-pin, would understand that in certain circumstances, the substitution of an attractive electrostatic interaction would have a small effect on lysozyme stability. It should be noted that Dao-pin was attempting to introduce a potentially favorable interaction, rather than eliminating an existing unfavorable electrostatic interaction. These two activities are fundamentally different. As is taught by Dao-pin, researches cannot know ahead of time if a potentially attractive electrostatic interaction would in fact be attractive. Conversely, the "discovery" part of eliminating unfavorable electrostatic interactions is determining which residues contribute to the unfavorable interaction. Once these unfavorable interactions are determined, it is straightforward to make an appropriate substitution. The present inventor made the discovery that the residues in the Asp 7, Asp 23 and Glu 9 triad contribute to an unfavorable electrostatic interaction, and determined that these residues could be

substituted with appropriate residues to eliminate the unfavorable interaction. The present inventor performed studies to determine that the substitution of a neutral or attractive electrostatic interaction of certain residues in Fn3fn10 resulted in a stabilizing electrostatic interaction in Fn3fn10.

Applicant asserts that he has provided adequate written description of a modified fibronectin type III tenth fibronectin unit (Fn3fn10) molecule comprising a stabilizing mutation of at least one residue involved in an unfavorable electrostatic interaction as compared to a wild-type Fn3, wherein the stabilizing mutation is a substitution of at least one of Asp 7, Asp 23 or Glu 9 with another amino acid residue. Applicant has disclosed in sufficient detail the relevant identifying characteristics that provide evidence that applicant was in possession of the claimed invention at the time the application was filed. Applicant requests that this rejection be withdrawn.

Claim 8 is rejected under 35 U.S.C. §112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. Claim 8 has been amended to recite that the three amino acid residues are substituted with other residues. Applicant requests that this rejection be withdrawn.

Claim Objections

Applicant acknowledges the examiner's statement that claims 4 and 7 are objected to as being dependent upon a rejected base claim, but would be allowable if rewritten in independent form.

CONCLUSION

Enclosed are checks in the amount of $250 for the Notice of Appeal fee and a check in the amount of $225 for the Petition for Extension of Time fee. Please apply any other charges or credits to deposit account 06-1050.

Respectfully submitted,

Date: 18 April 2005

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has received far more structural study than most). A selected protein or a member of a selected branch can be displayed using Chime.

5. **VAST** (*V*ector *A*lignment *S*earch *T*ool), a component of the National Center for Biotechnology Information (NCBI) Entrez system, reports a precomputed list of proteins of known structure that structurally resemble the query protein ("structure neighbors"). The VAST system uses the **Molecular Modeling Database (MMDB),** an NCBI-generated database that is derived from PDB coordinates but in which molecules are represented by connectivity graphs rather than sets of atomic coordinates. VAST displays the superposition of the query protein in its structural alignment with up to five other proteins using **Cn3D** [a molecular graphics program that displays MMDB files and that is publicly available for a variety of computer platforms (Table 8-4)] or with only one other protein using MAGE. VAST also reports a precomputed list of proteins that are similar to the query protein in sequence ("sequence neighbors") and provides links from a selected protein to several bibliographic databases including MedLine.

A number of other structural analysis/classification/comparison tools can be invoked from the "Other Sources" window of the Structure Explorer. The "Sequence Details" window provides the sequence of each chain in the structure and, for polypeptides, indicates the secondary structure of each of its residues.

4 ■ PROTEIN STABILITY

Incredible as it may seem, thermodynamic measurements indicate that *native proteins are only marginally stable entities under physiological conditions.* The free energy required to denature them is ~0.4 kJ · mol^{-1} of amino acid residues, so that 100-residue proteins are typically stable by only around 40 kJ · mol^{-1}. In contrast, the energy required to break a typical hydrogen bond is ~20 kJ · mol^{-1}. The various noncovalent influences to which proteins are subject—electrostatic interactions (both attractive and repulsive), hydrogen bonding (both intramolecular and to water), and hydrophobic forces—each have energetic magnitudes that may total thousands of kilojoules per mole over an entire protein molecule. Consequently, *a protein structure arises from a delicate balance among powerful countervailing forces.* In this section we discuss the nature of these forces and end by considering protein denaturation, that is, how these forces can be disrupted.

A. *Electrostatic Forces*

Molecules are collections of electrically charged particles and hence, to a reasonable degree of approximation, their interactions are determined by the laws of classical electrostatics (more exact calculations require the application of quantum mechanics). The energy of association, U, of two electric charges, q_1 and q_2, that are separated by the distance r is found by integrating the expression for Coulomb's law, Eq. [2.1], to determine the work necessary to separate these charges by an infinite distance:

$$U = \frac{kq_1q_2}{Dr} \qquad [8.1]$$

Here $k = 9.0 \times 10^9$ J · m · C^{-2} and D is the dielectric constant of the medium in which the charges are immersed (recall that $D = 1$ for a vacuum and, for the most part, increases with the polarity of the medium; Table 2-1). The dielectric constant of a molecule-sized region is difficult to estimate. For the interior of a protein, it is usually taken to be in the range 3 to 5 in analogy with the measured dielectric constants of substances that have similar polarities, such as benzene and diethyl ether.

Coulomb's law is only valid for point or spherically symmetric charges that are immersed in a medium of constant D. However, proteins are by no means spherical and their internal D values vary with position. Moreover, a protein in solution associates with mobile ions such as Na^+ and Cl^-, which modulate the protein's electrostatic potential. Consequently, calculating the electrostatic potential of a protein requires mathematically sophisticated and computationally intensive algorithms that are beyond the scope of this text. These methods are widely used to calculate the surface electrostatic potentials of proteins using a program called **GRASP** (for Graphical Representation and Analysis of Surface Properties) written by Anthony Nicholls, Kim Sharp, and Barry Honig. Figure 8-56 shows

FIGURE 8-56 A GRASP diagram of human growth hormone. The diagram shows the protein's surface colored according to its electrostatic potential, with its most negative areas dark red, its most positive areas dark blue, and its neutral areas white. The protein's orientation is the same as that in Fig. 8-47*b*. [Based on an X-ray structure by Alexander Wlodawer, National Cancer Institute, Frederick, Maryland. PDBid 1HGU.]

a GRASP diagram of human growth hormone in which the protein's surface is colored according to its electrostatic potential. Such diagrams are useful for assessing how a protein might associate with charged molecules such as other proteins, nucleic acids, and substrates. Similar computations are used to predict the pK's of protein surface groups, which can have significant application in the elucidation of an enzyme's mechanism of action (Section 15-1).

a. Ionic Interactions Are Strong but Do Not Greatly Stabilize Proteins

The association of two ionic protein groups of opposite charge is known as an **ion pair** or **salt bridge.** According to Eq. [8.1], the energy of a typical ion pair, say the carboxyl group of Glu and the ammonium group of Lys, whose charge centers are separated by 4.0 Å in a medium of dielectric constant 4, is $-86\ \text{kJ} \cdot \text{mol}^{-1}$ (one electronic charge $= 1.60 \times 10^{-19}$ C). However, free ions in aqueous solution are highly solvated, and the formation of a salt bridge has the entropic penalty of localizing the salt bridge's charged side chains. Consequently, the free energy of solvation of two separated ions is about equal to the free energy of formation of their unsolvated ion pair. *Ion pairs therefore contribute little stability toward a protein's native structure.* This accounts for the observations that although ~75% of charged residues occur in ion pairs, very few ion pairs are buried (unsolvated), and ion pairs that are exposed to the aqueous solvent tend to be but poorly conserved among homologous proteins.

b. Dipole–Dipole Interactions Are Weak but Significantly Stabilize Protein Structures

The noncovalent associations between electrically neutral molecules, collectively known as **van der Waals forces,** arise from electrostatic interactions among permanent and/or induced dipoles. These forces are responsible for numerous interactions of varying strengths between nonbonded neighboring atoms. (The hydrogen bond, a special class of dipolar interaction, is considered separately in Section 8-4B.)

Interactions among permanent dipoles are important structural determinants in proteins because many of their groups, such as the carbonyl and amide groups of the peptide backbone, have permanent dipole moments. These interactions are generally much weaker than the charge–charge interactions of ion pairs. Two carbonyl groups, for example, each with dipoles of 4.2×10^{-30} C · m (1.3 debye units) that are oriented in an optimal head-to-tail arrangement (Fig. 8-57*a*) and separated by 5 Å in a medium of dielectric constant 4, have a calculated attractive energy of only $-9.3\ \text{kJ} \cdot \text{mol}^{-1}$. Furthermore, these energies vary with r^{-3}, so they rapidly attenuate with distance. In α helices, however, the negative ends of the dipolar amide and carbonyl groups of the polypeptide backbone all point in the same direction (Fig. 8-11), so that their interactions and bond dipoles are additive (these groups, of course, also form hydrogen bonds, but here we are concerned with their residual electric fields). The α helix therefore has a significant dipole moment that is positive toward the N-terminus and negative toward the C-terminus. Consequently, *in the low dielectric constant core of a protein, dipole–dipole interactions significantly influence protein folding.*

A permanent dipole also induces a dipole moment on a neighboring group so as to form an attractive interaction (Fig. 8-57*b*). Such dipole–induced dipole interactions are generally much weaker than are dipole–dipole interactions.

Although nonpolar molecules are nearly electrically neutral, at any instant they have a small dipole moment resulting from the rapid fluctuating motions of their electrons. This transient dipole moment polarizes the electrons in a neighboring group, thereby giving rise to a dipole mo-

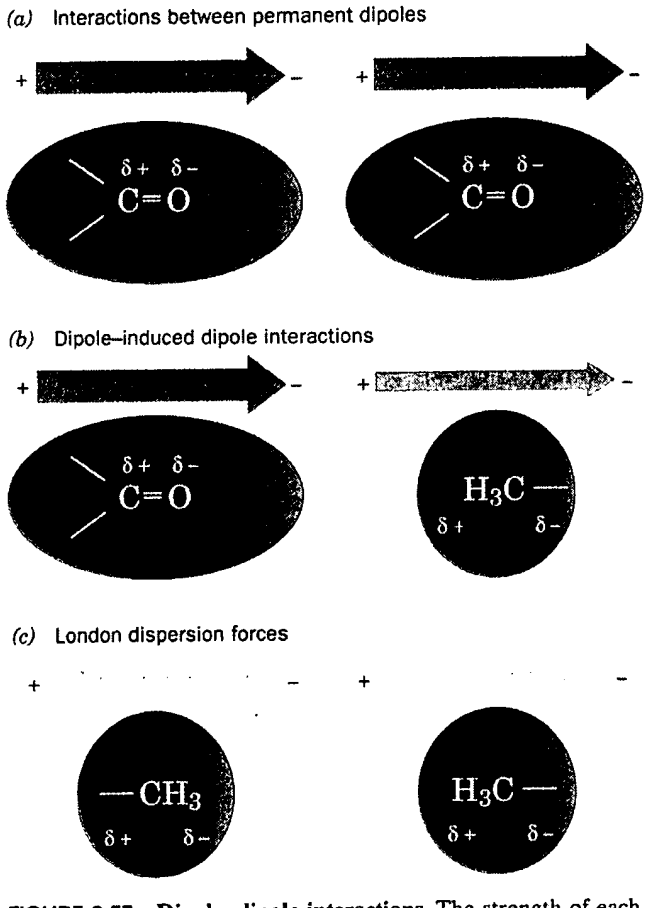

FIGURE 8-57 Dipole–dipole interactions. The strength of each dipole is represented by the thickness of the accompanying arrow. (*a*) Interactions between permanent dipoles. These interactions, here represented by carbonyl groups lined up head to tail, may be attractive, as shown, or repulsive, depending on the relative orientations of the dipoles. (*b*) Dipole–induced dipole interactions. A permanent dipole (here shown as a carbonyl group) induces a dipole in a nearby group (here represented by a methyl group) by electrostatically distorting its electron distribution (*shading*). This always results in an attractive interaction. (*c*) London dispersion forces. The instantaneous charge imbalance (*shading*) resulting from the motions of the electrons in a molecule (*left*) induces a dipole in a nearby group (*right*); that is, the motions of the electrons in neighboring groups are correlated. This always results in an attractive interaction.